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# Effect of $CO_2$ enrichment on the glucosinolate contents under different nitrogen levels in bolting stem of Chinese kale (*Brassica alboglabra* L.)*

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**Abstract:** The effects of $CO_2$ enrichment on the growth and glucosinolate (GS) concentrations in the bolting stem of Chinese kale (*Brassica alboglabra* L.) treated with three nitrogen (N) concentrations (5, 10, and 20 mmol/L) were investigated. Height, stem thickness, and dry weights of the total aerial parts, bolting stems, and roots, as well as the root to shoot ratio, significantly increased as $CO_2$ concentration was elevated from 350 to 800 μl/L at each N concentration. In the edible part of the bolting stem, 11 individual GSs were identified, including 7 aliphatic and 4 indolyl GSs. GS concentration was affected by the elevated $CO_2$ concentration, N concentration, and $CO_2$×N interaction. At 5 and 10 mmol N/L, the concentrations of aliphatic GSs and total GSs significantly increased, whereas those of indolyl GSs were not affected, by elevated atmospheric $CO_2$. However, at 20 mmol N/L, elevated $CO_2$ had no significant effects on the concentrations of total GSs and total indolyl GSs, but the concentrations of total aliphatic GSs significantly increased. Moreover, the bolting stem carbon (C) content increased, whereas the N and sulfur (S) contents decreased under elevated $CO_2$ concentration in the three N treatments, resulting in changes in the C/N and N/S ratios. Also the C/N ratio is not a reliable predictor of change of GS concentration, while the changes in N and S contents and the N/S ratio at the elevated $CO_2$ concentration may influence the GS concentration in Chinese kale bolting stems. The results demonstrate that high nitrogen supply is beneficial for the growth of Chinese kale, but not for the GS concentration in bolting stems, under elevated $CO_2$ condition.

**Key words:** Carbon dioxide ($CO_2$), *Brassica alboglabra*, Nitrogen (N), Growth, Bolting stem, Aliphatic glucosinolates, Indolyl glucosinolates, Carbon/nitrogen ratio (C/N), Nitrogen/sulfur ratio (N/S)

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## INTRODUCTION

Epidemiological studies show that there is a negative relationship between Brassicaceae vegetable intake and the risk of a number of cancers (Wattenberg, 1993; Kohlmeier and Su, 1997; Price *et al*., 1998). Recently, it has been widely recognized that some of the cancer-chemoprotective activities in these vegetables are attributable to their contents of glucosinolates (GSs) (Zhao *et al*., 1992; Wattenberg, 1993; Tawfiq *et al*., 1995; Fahey *et al*., 1997; Rosa *et al*., 1997; Holst and Williamson, 2004) (Fig.1). GSs are amino acid-derived secondary compounds, a characteristic of dicotyledonous plants. So far, more than 20 GSs have been identified in Brassicaceae family (Rodman, 1991). They can be grouped into aliphatic, aromatic, and indolyl GSs according to the amino acid, from which they are derived (Louda and

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Mole, 1991; Halkier and Du, 1997). Besides the health-promoting properties, GSs also play an important role in plant defense against insects and herbivores, and are utilized as special flavors in the food industry (Fenwick *et al*., 1983; Chew, 1988; Gijzen *et al*., 1989; Baik *et al*., 2003).

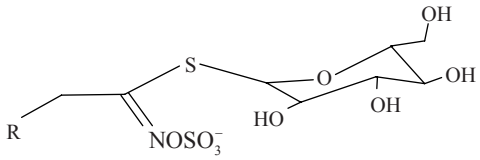


**Fig.1 General structure of glucosinolates (GSs)**

It is widely accepted that GS content is affected by environmental factors including climatic conditions, nutritional availability, and agronomic practices, in addition to genetic characteristics (Fenwick *et al*., 1989). Presently, cancer incidence and global climate change are major topical issues. Because GSs exhibit cancer-chemoprotective activities, many researches are focused on the effect of climatic conditions on changes in GS content in vegetables (Schreiner, 2005). The rise in atmospheric carbon dioxide ($CO_2$) concentration is one of the most prominent climatic changes in recent decades (IPCC, 2007). Climate simulations indicate that the atmospheric $CO_2$ concentration is expected to reach 700 μl/L by the end of this century, which is double as much as the current $CO_2$ concentration (Caswell, 2004). Increasing atmospheric $CO_2$ concentration may affect natural ecosystems by directly influencing plant growth and photochemistry due to an increased photosynthetic rate, especially in $C_3$ plants (Islam *et al*., 1996; Kim *et al*., 2001; Das *et al*., 2002). In spite of increased plant growth under elevated $CO_2$ concentrations (Bazzaz, 1990; Mooney *et al*., 1991; Amthor, 2001), aerial plant parts accumulate generally less nitrogen (N), and carbon (C)/N ratio increases (Baxter *et al*., 1994; Epron *et al*., 1996), which could influence plant secondary metabolites synthesis and concentration. GSs, as N- and C-containing secondary metabolites, might be affected by atmospheric $CO_2$ enrichment owing to changes in the plant's C supply and N content (Cotrufo *et al*., 1998). Moreover, Habash *et al*. (1995) observed that synthesis of the amino acid precursors of GSs from triosephosphates increased at an elevated $CO_2$ concentration. However, Karowe *et al*.(1997) reported that total foliar GS content in mustard decreased significantly under elevated $CO_2$ conditions. This conflict might reflect a species-specific response to elevated $CO_2$ concentration (Karowe *et al*., 1997). An increasing number of studies indicate that N availability can have a large impact on the plant response to elevated $CO_2$ concentration (Kimball *et al*., 1995; 2002). Moreover, N application is one of the most important nutrient factors that significantly affect GS synthesis and content (Schnug, 1989; Zhao *et al*., 1994; Ahmad *et al*., 2007). In oilseed rape (*Brassica napus* L.), the GS content in the seed decreased with the higher N supply in sulfur (S)-deficient soil, but increased in S-sufficient soil (Zhao *et al*., 1994). However, in broccoli sprouts (*Brassica oleracea* var. *italica*), N fertilization has a negative effect on GS content, even at a very low concentration (Aires *et al*., 2006).

Therefore, it is logical to take N nutrition into account when investigating the effects of $CO_2$ enrichment on GS content. At present, limited information is available on the effect of elevated atmospheric $CO_2$ in combination with N availability on GS content in brassicaceous vegetables. It is not clear whether GS content changes consistently at different N levels. In this study, we determined the interactive effects of elevated $CO_2$ concentration and N availability on the contents of individual and total GSs in the edible part of the bolting stem of Chinese kale, which is a nutritionally healthy vegetable belonging to the Brassicaceae (Cruciferae) family and rich in GSs, and has spread quickly in southeastern China, Taiwan region, and Japan since the last decade (He *et al*., 2002).

## MATERIALS AND METHODS

### Plant growth

Seeds of Chinese kale (*Brassica alboglabra* L. var. *Sijicutiao*) were sown in vermiculite and germinated in a greenhouse with computer-controlled growth conditions on Huajiachi campus of Zhejiang University, Hangzhou, China. The growth conditions in the greenhouse were constant day/night temperature of 23/18 °C and the natural photoperiod. After two weeks, the seedlings were irrigated with nutrient solution containing 5 mmol N/L. Four weeks later, healthy seedlings in which the third true leaf had emerged were transplanted to 1.8-L pots containing

nutrient solution with 10 mmol N/L by being fixed in a foam cavity with sponge. Each pot contained two seedlings and was covered with black plastic foil to prevent algal growth and evaporation. All the pots were transferred to four growth chambers with 65% relative humidity, constant day/night temperature of 23/18 °C, and 500 μmol/($m^2$·s) photosynthetically-active radiation for 16 h/d. One week after transplanting, plants were treated with 5.0 mmol N/L (low N concentration), 10 mmol N/L (medium N concentration), or 20 mmol N/L (high N concentration) (Chen *et al*., 2005), and were grown under either ambient $CO_2$ [(350±20) μl/L, denoted as A] or elevated $CO_2$ [(800±20) μl/L, denoted as E). The N was supplied as $NH_4NO_3$ and the basic nutrient solution contained 1 mmol/L $K_2HPO_4$, 4 mmol/L KCl, 3 mmol/L $CaCl_2$, 2 mmol/L $MgSO_4·7H_2O$, 36 μmol/L ethylene diamine tetraacetic acid (EDTA)-Fe, 46.4 μmol/L $H_3BO_3$, 9.07 μmol/L $MnCl_2·4H_2O$, 0.765 μmol/L $ZnSO_4·7H_2O$, 0.3 μmol/L $CuSO_4·5H_2O$, and 0.09 μmol/L $H_2MoO_4·H_2O$ (Hoagland and Arnon, 1938). $CO_2$ was supplied from gas tanks for the elevated $CO_2$ concentration treatment. There were two replicate chambers per $CO_2$ concentration and three replicate pots per N treatment in each chamber. The position of every pot was rotated randomly when the solutions were renewed every three days. To avoid a potential chamber effect, the pots were switched with those from the other chamber with the same $CO_2$ concentration every week. The culture solutions were continuously aerated and adjusted to pH 6.0 using diluted NaOH or HCl every day until harvest. After treated for 35 d, the selected growth parameters were measured, and every plant was separated into different parts, weighed, and lyophilized. The bolting stems were ground into a powder and stored in a desiccator at −20 °C prior to C, N, S, and GS analyses.

**Extract preparation for glucosinolate analysis**

Extracts for GS analysis were prepared according to the method of Kiddle *et al*.(2001) with some modifications. Triplicate samples (0.1 g) of freeze-dried powder were each weighed in 5 ml tubes, and crude GSs were extracted with 1.5 ml 70% (v/v) methanol at 75 °C for 10 min in a water bath. The mixture was centrifuged at 5000×*g* for 10 min at 4 °C and the supernatant was decanted into another tube. The extraction was repeated twice from residues using the same procedure. The three supernatants were combined to give a final extract volume of 5 ml. 2 ml of each GS extract was added to a mini-column filled with diethylaminoethanol (DEAE) Sephadex A-25 (80 mg as dry matter) (170170-01, Amersham Biosciences, Sweden) activated with 0.5 mol/L pyridine acetate, and desulfated by sulfatase (S9626, Sigma-Aldrich Co., MO, USA). After reaction at room temperature overnight (16 h), the desulfated glucosinolates (desulfoGSs) were eluted with 2 ml deionized water and stored at −20 °C prior to high-performance liquid chromatography (HPLC) analysis. 2-PropenylGS (sinigrin, S1647, Sigma-Aldrich Co., MO, USA) was used as an external standard for GS quantitative analysis.

**High performance liquid chromatography**

The desulfated extract (20 μl) was analyzed by HPLC (Beckman Coulter System Gold HPLC, Beckman, USA) using a Hypersil ODS2 column (250 mm×4.6 mm, 5 μm; Elite, China) with a Beckman Ultrasphere ODS guard column (45 mm×4.6 mm, 5 μm; Beckman, USA). The wavelength of the ultraviolet detector was set at 227 nm. The mobile phase was a mixture of deionized water (A) and acetonitrile (B) and ran at a flow rate of 1 ml/min. The elution program consisted of a linear gradient from 0 to 20% (B) in 18 min and constant 20% (B) for a further 16 min, then the column was eluted with 100% (B) for 5 min and equilibrated with 0 (B) for 6 min prior to the injection of the next sample (Macfarlane-Smith and Griffiths, 1988).

**Mass spectrometry analysis**

The separated compounds were identified according to the mass spectrometry (MS) data obtained by a liquid chromatography-mass spectrometry data (LC-MSD) system (Agilent 1100 LC/MSD, Agilent Co., USA). The conditions used for the electrospray source were ionspray mode, positive; capillary voltage, 4 kV; nebulizer pressure, 42184.8 Pa; fragment voltage, 100 V; curtain gas, nitrogen; drying gas flow, 13 L/min; desolvation gas temperature, 350 °C. Each individual desulfoGS was identified according to their $(M+H)^+$, $(M+Na)^+$, $(M+K)^+$, and (M-glucosyl+H)$^+$ in the MS.

**Analyses of bolting stem carbon, nitrogen, and sulfur contents**

Bolting stem C content was determined by titration after digestion by $H_2SO_4$ and potassium permanganate (Lu, 1999). Bolting stem N content was determined titrimetrically using the Kjeldahl procedure with salicylic acid, sodium thiosulfate, and zinc as catalysts (Pruden *et al*., 1985). For measuring the S content, 0.1 g aliquot of the ground materials was digested with $HNO_3$ and $HClO_4$. The S content was determined using an inductively coupled plasma atomic emission spectrometer (ICP-MS; Agilent, 7500a, USA) (Lu, 1999).

**Statistical analysis**

Two-way analysis of variance (ANOVA) was performed to determine the main effects (N and $CO_2$ treatments) and their interactions with a significance level of $P<0.05$. The normality of data and the homogeneity of variances were verified by Shapiro-Wilk test and Bartlett test, respectively, before using ANOVA. Differences between means were analyzed by Fisher's protected least significant difference (LSD) procedure. All statistical analysis procedures were performed by using SPSS for Windows version 12.0 (SPSS, Chicago, IL, USA).

## RESULTS

**Plant growth**

Elevated $CO_2$ concentration significantly increased plant height, stem thickness, dry weights of the total aerial parts, bolting stems, and roots, and the root-to-shoot ratio, compared with those in the ambient $CO_2$ treatment (Table 1). Regardless of N concentration, the height, stem thickness, dry weights of the total aerial parts, bolting stems, and roots, and root-to-shoot ratio increased by 15.64%, 11.79%, 11.91%, 15.03%, 16.34%, and 3.90%, respectively, with elevated $CO_2$ concentration. Nitrogen levels also significantly affected each growth parameter (Table 1). The 10 mmol N/L solution significantly increased the height, stem thickness, and dry weights of the total aerial parts, bolting stems, and roots, compared with those in the 5 mmol N/L solution in both $CO_2$ regimes. However, there was no significant difference between the 10 and 20 mmol N/L solution treatments for the above parameters in both $CO_2$ conditions, except that height and dry weight of the total aerial parts differed significantly between the two N treatments at the elevated $CO_2$ concentration. The root-to-shoot ratio did not differ significantly among the three N concentrations at the ambient $CO_2$ concentration, but there was a significant difference between the 5 and 10 mmol/L N concentrations at the elevated $CO_2$ concentration. Moreover, there were significant $CO_2$×N interactions for plant height ($P<0.01$) and dry weights of the total aerial parts ($P<0.01$) and roots ($P<0.05$), but not for bolting stem thickness, dry weight of the bolting stem, or root-to-shoot ratio (Table 1).

**Glucosinolate content**

A typical HPLC profile of desulfoGSs in the bolting stem is shown in Fig.2. Eleven individual GSs

**Table 1 Effects of elevated $CO_2$ concentration on height, stem thickness, dry weights of aerial parts, bolting stems, and roots, and root-to-shoot ratio of Chinese kale at three nitrogen (N) concentrations**

| $CO_2$ (μl/L) | N (mmol/L) | Height (cm) | Stem thickness (cm) | Dry weight (g/plant) | | | Root-to-shoot ratio |
|---|---|---|---|---|---|---|---|
| | | | | Total aerial part | Bolting stem | Root | |
| 350 | 5 | 26.13±3.00$^{d}$ | 0.72±0.02$^{d}$ | 4.84±0.10$^{e}$ | 1.63±0.10$^{d}$ | 0.45±0.03$^{d}$ | 0.093±0.006$^{c}$ |
| | 10 | 29.13±2.59$^{c}$ | 0.82±0.09$^{bc}$ | 6.30±0.12$^{c}$ | 2.24±0.08$^{b}$ | 0.61±0.02$^{b}$ | 0.096±0.005$^{abc}$ |
| | 20 | 28.25±1.98$^{cd}$ | 0.81±0.04$^{c}$ | 6.27±0.07$^{c}$ | 2.29±0.13$^{b}$ | 0.60±0.02$^{b}$ | 0.095±0.004$^{bc}$ |
| 800 | 5 | 32.44±2.22$^{b}$ | 0.77±0.04$^{cd}$ | 5.38±0.17$^{d}$ | 1.96±0.07$^{c}$ | 0.51±0.03$^{c}$ | 0.095±0.004$^{bc}$ |
| | 10 | 34.88±1.48$^{a}$ | 0.88±0.08$^{ab}$ | 6.97±0.13$^{b}$ | 2.53±0.16$^{a}$ | 0.70±0.02$^{a}$ | 0.100±0.002$^{a}$ |
| | 20 | 29.25±1.58$^{c}$ | 0.89±0.07$^{a}$ | 7.14±0.16$^{a}$ | 2.60±0.10$^{a}$ | 0.71±0.03$^{a}$ | 0.100±0.007$^{ab}$ |
| Source of variance | | | | | | | |
| $CO_2$ | | *** | ** | *** | *** | *** | * |
| N | | *** | *** | *** | *** | *** | * |
| $CO_2$×N | | ** | NS | ** | NS | * | NS |

Data followed by the same superscript letter(s) indicate no significant difference at the $P<0.05$ level. Values are the mean±*SD*. Significance levels indicated by two-way ANOVA: NS, not significant; $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

were identified according to the elution order from the HPLC column and confirmed by electrospray ionization mass spectrometry (ESI-MS) analysis based on their MS data. All GSs were identified by analyzing the chemical structure of the aglucone chain R and described according to the trivial names that have been popularly used for decades (Fig.1 and Table 2). Seven aliphatic GSs comprising glucoiberin, progoitrin, sinigrin, glucoraphanin, glucoalyssin, gluconapin, and glucoerucin, and four indolyl GSs consisting of 4-hydroxyglucobrassicin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin, were identified.

The major GSs were gluconapin, sinigrin, glucoraphanin, and glucoiberin, which constituted about 54.48%, 8.80%, 8.63%, and 5.93%, respectively, of the total GS concentration on average. The proportions of the other seven individual GSs were less than 5% on average. The total GS concentration in each treatment was expressed as the sum of the 11 identified individual GS species (Fig.3). The total GS content ranged 4.82~6.41 μmol/g DW (dry weight). $CO_2$ concentration significantly affected the concentrations of total GSs, total aliphatic GSs, and all individual aliphatic GSs except glucoerucin, but not the concentrations of total or individual indolyl GSs (Table 3 and Fig.3). Under elevated $CO_2$, the total GS concentration increased in the 5 and 10 mmol N/L treatments by 15.59% and 18.01%, respectively, compared with those at ambient $CO_2$. However, elevated $CO_2$ did not affect the total GS concentration at 20 mmol N/L. In the 5 and 10 mmol N/L solution treatments, all individual aliphatic GSs increased under elevated $CO_2$ compared with the ambient $CO_2$

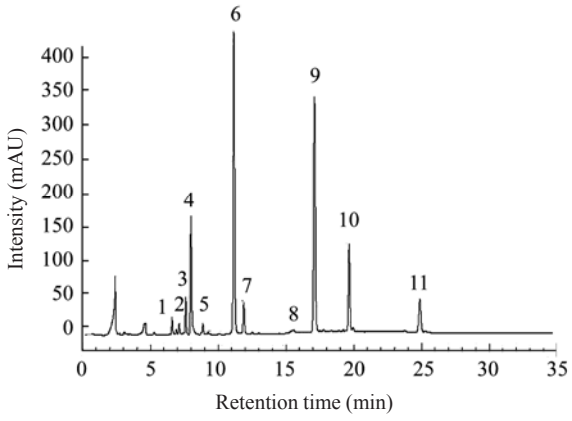


**Fig.2 Typical HPLC elution profile of desulfated glucosinolates in bolting stem of Chinese kale**

Peak numbers refer to GSs listed in Table 2

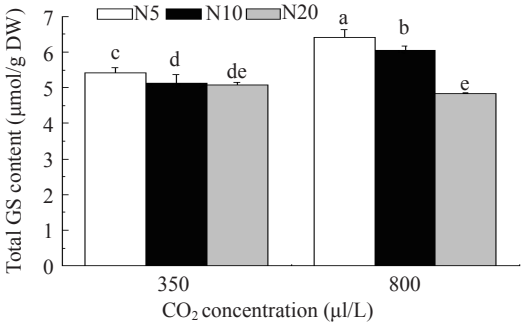


**Fig.3 Comparison of the total GS concentration in bolting stems of Chinese kale grown at ambient $CO_2$ (350 μl/L) and elevated $CO_2$ (800 μl/L) concentrations under three nitrogen (N) concentrations**

N5: 5 mmol N/L; N10: 10 mmol N/L; N20: 20 mmol N/L. Columns with the same letter(s) indicate no significant difference at the $P<0.05$ level. The bars represent the standard error

**Table 2 Desulfated glucosinolates identified in bolting stems of Chinese kale**

| No.* | Retention time (min) | Side-chain structure | Trivial name | Desulfated molecular weight | Response factor† |
|---|---|---|---|---|---|
| 1 | 6.58 | $CH_3$-SO-$(CH_2)_3$- | Glucoiberin | 343 | 1.07 |
| 2 | 7.10 | $CH_2$=CHCH(OH)$CH_2$- | Progoitrin | 309 | 1.09 |
| 3 | 7.59 | $CH_2$=$CHCH_2$- | Sinigrin | 279 | 1.00 |
| 4 | 7.98 | $CH_3$-SO-$(CH_2)_4$- | Glucoraphanin | 357 | 1.07 |
| 5 | 9.24 | $CH_3$-SO-$(CH_2)_5$- | Glucoalyssin | 371 | 1.07 |
| 6 | 11.13 | $CH_2$=CH-$(CH_2)_2$- | Gluconapin | 293 | 1.11 |
| 7 | 11.86 | Indole-(4-OH)-3-$CH_2$- | 4-Hydroxyglucobrassicin | 384 | 0.28 |
| 8 | 15.54 | $CH_3$-S-$(CH_2)_4$- | Glucoerucin | 341 | 1.00‡ |
| 9 | 17.08 | Indole-3-$CH_2$- | Glucobrassicin | 368 | 0.29 |
| 10 | 19.64 | Indole-(4-$OCH_3$)-3-$CH_2$- | 4-Methoxyglucobrassicin | 398 | 0.25 |
| 11 | 24.86 | Indole-($OCH_3$)-3-$CH_2$- | Neoglucobrassicin | 398 | 0.20 |

* Numbering is based on the elution order of desulfated glucosinolates from HPLC; † The response factors relative to the standard sinigrin were experimentally determined with HPLC by the International Organization for Standardization (ISO 9167-1) in 1992 for individual GS content in rapeseed; ‡ Not yet determined by the ISO

treatment, whereas individual indolyl GSs were not affected by the elevated $CO_2$ concentration. However, in the 20 mmol N/L treatment under the elevated $CO_2$ condition, the total aliphatic GS concentration decreased significantly ($P$<0.01), while there was no significant effect on total indolyl GS content ($P$>0.05), resulting in a slight decrease in total GS concentration ($P$>0.05). Nitrogen concentration also significantly affected the concentrations of total GSs, total aliphatic GSs, individual aliphatic GSs except glucoerucin, total indolyl GSs, and individual indolyl GSs except 4-methoxyglucobrassicin (Tables 3 and 4). The 5 mmol N/L treatment increased the total aliphatic GS concentration compared with the 10 and 20 mmol N/L treatments, but there was no significant difference in the effects of the 10 and 20 mmol N/L treatments at ambient $CO_2$. However, under the elevated $CO_2$ concentration, the total aliphatic GS concentration decreased significantly with increased N supply. Total indolyl GS concentration increased in the 20 mmol/L N treatment compared with that in the 5 mmol N/L treatment at both $CO_2$ concentrations, whereas the difference between the 10 and 20 mmol N/L treatments was not significant at the ambient $CO_2$ concentration but was significant under the elevated $CO_2$ concentration. Moreover, there were significant $CO_2$×N interactions for total GS concentration ($P$<0.001), total aliphatic GS concentration ($P$<0.001), and concentrations of all individual aliphatic GSs ($P$<0.01) except glucoerucin, but not for the concentrations of total indolyl GSs or individual indolyl GSs ($P$>0.05) except neoglucobrassicin.

**Bolting stem carbon, nitrogen, and sulfur contents**

With elevated $CO_2$, the C content in the bolting stem increased in the 5, 10, and 20 mmol N/L treatments by 11.38%, 13.62%, and 10.46%, respectively, relative to the ambient $CO_2$ concentration (Table 5). The effect of $CO_2$ on C content was strongly significant ($P$<0.001), but the effects of N concentration and $CO_2$×N interactions were not significant ($P$>0.05). The N content in bolting stem increased significantly with increasing N concentration in the nutrient solution under the same $CO_2$ regime. In contrast, the N content decreased in the three N treatments under the enriched $CO_2$ concentration compared with that of the ambient $CO_2$ treatment (Table 5). The decreases in N content in the 5, 10, and 20 mmol N/L treatments were 10.97%, 13.27%, and 4.59%, respectively. There were significant $CO_2$×N interactions for the C/N ratio (Table 5). The C/N ratio at the three N concentrations all increased under the elevated $CO_2$ concentration by 25.13%, 31.20%, and 15.73%, respectively. There were significant N concentration ($P$<0.01) and $CO_2$ concentration ($P$<0.001) effects on S content, but $CO_2$×N interactions were not significant for S content. Under the elevated $CO_2$ concentration, S content decreased in all of the N treatments. Because of the decreases in both N and S contents, the N/S ratio changed. The N/S ratio was significantly affected by $CO_2$ concentration ($P$<0.05), N concentration ($P$< 0.001), and $CO_2$×N interactions ($P$<0.01). Under the elevated $CO_2$ concentration, the N/S ratio decreased significantly in the 5 and 10 mmol N/L treatments ($P$<0.05), but the decrease in N and S

**Table 3 Effect of $CO_2$ concentration on the individual and total aliphatic GS concentrations in bolting stems of Chinese kale at three nitrogen (N) concentrations**

| $CO_2$ (μl/L) | N (mmol/L) | GS concentration (μmol/g DW) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | GIB | PRO | GRA | SIN | GAL | GNP | GRU | Total |
| 350 | 5 | 0.17±0.02$^{d}$ | 0.13±0.02$^{c}$ | 0.15±0.01$^{d}$ | 0.51±0.02$^{b}$ | 0.10±0.01$^{b}$ | 3.62±0.11$^{b}$ | 0.05±0.01$^{b}$ | 4.71±0.12$^{c}$ |
| | 10 | 0.31±0.01$^{c}$ | 0.14±0.01$^{c}$ | 0.46±0.08$^{c}$ | 0.40±0.08$^{bc}$ | 0.10±0.01$^{b}$ | 2.89±0.12$^{d}$ | 0.06±0.01$^{ab}$ | 4.36±0.22$^{d}$ |
| | 20 | 0.34±0.01$^{b}$ | 0.25±0.01$^{b}$ | 0.88±0.06$^{a}$ | 0.35±0.02$^{c}$ | 0.23±0.03$^{a}$ | 2.17±0.03$^{e}$ | 0.04±0.01$^{b}$ | 4.27±0.08$^{d}$ |
| 800 | 5 | 0.46±0.01$^{a}$ | 0.38±0.07$^{a}$ | 0.18±0.02$^{d}$ | 0.64±0.12$^{a}$ | 0.21±0.05$^{a}$ | 3.85±0.14$^{a}$ | 0.07±0.03$^{a}$ | 5.79±0.14$^{a}$ |
| | 10 | 0.35±0.03$^{b}$ | 0.16±0.04$^{c}$ | 0.49±0.03$^{c}$ | 0.70±0.04$^{a}$ | 0.15±0.02$^{b}$ | 3.39±0.17$^{c}$ | 0.06±0.00$^{ab}$ | 5.31±0.09$^{b}$ |
| | 20 | 0.33±0.02$^{bc}$ | 0.25±0.03$^{b}$ | 0.57±0.02$^{b}$ | 0.35±0.00$^{c}$ | 0.22±0.02$^{a}$ | 2.16±0.03$^{e}$ | 0.04±0.01$^{b}$ | 3.93±0.01$^{e}$ |
| Source of variance | | | | | | | | | |
| $CO_2$ | | *** | *** | ** | *** | ** | *** | NS | *** |
| N | | NS | *** | *** | *** | *** | *** | NS | *** |
| $CO_2$×N | | *** | *** | *** | ** | ** | ** | NS | *** |

GIB: glucoiberin; PRO: progoitrin; GRA: glucoraphanin; SIN: sinigrin; GAL: glucoalyssin; GNP: gluconapin; GRU: glucoerucin. Data followed by the same superscript letter(s) indicate no significant difference at $P$<0.05 level. Values are mean±*SD*. Significance levels indicated by two-way ANOVA: NS, not significant; $^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001

**Table 4 Effect of $CO_2$ concentration on the individual and total indolyl GS concentrations in bolting stems of Chinese kale at three nitrogen (N) concentrations**

| $CO_2$ (μl/L) | N (mmol/L) | GS concentration (μmol/g DW) | | | | |
|---|---|---|---|---|---|---|
| | | 4HGB | GBS | 4MGB | NGBS | Total |
| 350 | 5 | 0.01±0.00$^{bc}$ | 0.18±0.04$^{b}$ | 0.26±0.03$^{a}$ | 0.24±0.01$^{b}$ | 0.70±0.06$^{cd}$ |
| | 10 | 0.02±0.01$^{a}$ | 0.25±0.01$^{a}$ | 0.25±0.03$^{a}$ | 0.25±0.03$^{b}$ | 0.77±0.07$^{bc}$ |
| | 20 | 0.02±0.00$^{a}$ | 0.28±0.00$^{a}$ | 0.27±0.01$^{a}$ | 0.25±0.01$^{b}$ | 0.81±0.01$^{ab}$ |
| 800 | 5 | 0.01±0.00$^{c}$ | 0.14±0.04$^{b}$ | 0.25±0.03$^{a}$ | 0.23±0.02$^{b}$ | 0.62±0.07$^{d}$ |
| | 10 | 0.02±0.00$^{ab}$ | 0.25±0.01$^{a}$ | 0.25±0.03$^{a}$ | 0.24±0.04$^{b}$ | 0.75±0.05$^{bc}$ |
| | 20 | 0.02±0.00$^{a}$ | 0.28±0.01$^{a}$ | 0.27±0.01$^{a}$ | 0.33±0.02$^{a}$ | 0.89±0.04$^{a}$ |
| Source of variance | | | | | | |
| $CO_2$ | | NS | NS | NS | NS | NS |
| N | | *** | *** | NS | ** | *** |
| $CO_2$×N | | NS | NS | NS | * | NS |

4HGB: 4-hydroxyglucobrassicin; GBS: glucobrassicin; 4MGB: 4-methoxyglucobrassicin; NGBS: neoglucobrassicin. Data followed by the same superscript letter(s) indicate no significant difference at $P<0.05$ level. Values are mean±*SD*. Significance levels indicated by two-way ANOVA: NS, not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

**Table 5 Effect of $CO_2$ concentration on the carbon (C), nitrogen (N), and sulfur (S) contents, C/N ratio, and N/S ratio in bolting stems of Chinese kale at three N concentrations**

| $CO_2$ (μl/L) | N (mmol/L) | C content (%) | N content (%) | S content (%) | C/N ratio | N/S ratio |
|---|---|---|---|---|---|---|
| 350 | 5 | 30.88±0.81$^{b}$ | 3.58±0.05$^{e}$ | 0.81±0.01$^{a}$ | 8.62±0.10$^{c}$ | 4.42±0.09$^{d}$ |
| | 10 | 30.87±1.00$^{b}$ | 4.44±0.08$^{c}$ | 0.85±0.02$^{ab}$ | 6.95±0.17$^{d}$ | 5.21±0.15$^{b}$ |
| | 20 | 31.81±0.37$^{b}$ | 5.06±0.11$^{a}$ | 0.83±0.02$^{b}$ | 6.29±0.18$^{e}$ | 6.09±0.19$^{a}$ |
| 800 | 5 | 34.39±0.12$^{a}$ | 3.19±0.03$^{f}$ | 0.77±0.02$^{b}$ | 10.79±0.12$^{a}$ | 4.16±0.12$^{e}$ |
| | 10 | 35.08±0.14$^{a}$ | 3.85±0.08$^{d}$ | 0.81±0.02$^{c}$ | 9.12±0.43$^{b}$ | 4.77±0.11$^{c}$ |
| | 20 | 35.13±0.47$^{a}$ | 4.82±0.10$^{b}$ | 0.77±0.02$^{c}$ | 7.28±0.25$^{d}$ | 6.27±0.17$^{a}$ |
| Source of variance | | | | | | |
| $CO_2$ | | *** | *** | *** | *** | * |
| N | | NS | *** | ** | *** | *** |
| $CO_2$×N | | NS | * | NS | ** | ** |

Data followed by the same superscript letter(s) indicate no significant difference at $P<0.05$ level. Values are mean±*SD*. Significance levels indicated by two-way ANOVA: NS, not significant; * $P<0.05$; ** $P<0.01$; *** $P<0.001$

contents at the ambient and elevated $CO_2$ concentrations did not differ significantly in the 20 mmol N/L treatment.

## DISCUSSION

Little is known about the interactive effect of $CO_2$ enrichment and N availability on GS content in vegetables. In the present study, the effect of an elevated $CO_2$ concentration at three different N concentrations (5, 10, and 20 mmol/L) on GS content in the bolting stem of Chinese kale was investigated. We observed that the $CO_2$ concentration and N concentration in the nutrient solution showed significant interactive effects on the height and dry weights of the aerial parts, bolting stems, and roots. The maximum stem thickness and dry weights of the aerial parts, bolting stems, and roots were obtained at 20 mmol N/L under the elevated $CO_2$ condition, which confirms the importance of N availability in determining the vegetable's response to elevated $CO_2$. Similar conclusions have also been reported for the grass *Bromus mollis*, rice, wheat, and tomato seedlings (Larigauderie *et al*., 1988; Kim *et al*., 2001; Kobayashi *et al*., 2001; Li *et al*., 2007).

In this study, 11 individual GSs were detected in bolting stems of Chinese kale, of which the major GSs were gluconapin, sinigrin, glucoiberin, and glucoraphanin. These results are in agreement with a previous study on Chinese kale (La *et al*., 2008). Glucoraphanin, sinigrin, glucoiberin, and glucobrassicin are reported to be the most important GSs for the hydrolysis products serving as the most powerful agents protecting human and animal cells against carcinogenesis (Bones and Rossiter, 1996, Fahey *et*

*al*., 1997; Nilsson *et al*., 2006). We detected all the GSs in bolting stem, and the concentrations of sinigrin, glucoiberin, and glucobrassicin were similar to those reported for broccoli (Kushad *et al*., 1999; Padilla *et al*., 2007). A high concentration of progoitrin in vegetables is a latent problem because it causes goiter and other harmful effects on animals, such as depressed growth, poor egg production, and liver damage (Heaney and Fenwick, 1995). However, there is no evidence that *Brassica* consumption has any goitrogenic effects on humans (Mithen *et al*., 2000) and also there is no normative limit issued yet for progoitrin concentration in vegetables. Fortunately, the concentration of progoitrin detected in the edible part of Chinese kale was relatively low, ranging 0.12~0.37 μmol/g DW.

The elevated $CO_2$ concentration increased the total GS concentration as a result of a strong increase in aliphatic GSs, whereas there was no significant effect on the concentrations of indolyl GSs at 5 or 10 mmol N/L, compared with those at the ambient $CO_2$ concentration. This is in agreement with a previous study on broccoli inflorescences (Schonhof *et al*., 2007). However, at 20 mmol N/L, the difference between the ambient and elevated $CO_2$ treatments was not significant.

To our knowledge, no corresponding previous study has reported on the interactive effect of $CO_2$ and N concentrations on GS content. Bryant *et al*.(1983) advanced the carbon/nutrient balance hypothesis to predict the change of N- and C-containing compounds under elevated $CO_2$ conditions. These authors pointed out that, under an elevated $CO_2$ concentration, because of the increased C supply and N limitation, the concentrations of N- and C-containing compounds increased. Studies on the changes in condensed tannin, soluble phenolic polymer, and GS concentrations in the oilseed rape (*Brassica napus*) leaves under $CO_2$ enrichment are consistent with this hypothesis (Peñuelas and Estiarte, 1998; Himanen *et al*., 2008). However, some studies of the effect of $CO_2$ enrichment on GS content are not consistent with the prediction of the carbon/nutrient balance hypothesis (Karowe *et al*., 1997; Reddy *et al*., 2004; Schonhof *et al*., 2007). In the present study, under the elevated $CO_2$ condition, the C content in bolting stem of Chinese kale increased while the N content decreased in all of the three N treatments, which resulted in an increase in the C/N ratio; however, GS concentration did not change consistently with the increase in C/N ratio. Clearly, the change in C/N ratio was not a reliable predictive tool for the change in GS composition and concentration in Chinese kale bolting stems.

N and S are not only two essential elements that are constituents of amino acids, but also the main factors that affect GS content in bolting stems of Chinese kale. Under the elevated $CO_2$ concentration, besides the decrease in N content, S content simultaneously decreased in all of the N treatments. However, in broccoli inflorescences, N content decreased while the S content was unaffected by a rise in $CO_2$ concentration because of the unchanged fresh and dry weights of broccoli between $CO_2$ regimes (Schonhof *et al*., 2007). In young pedunculate oak (*Quercus robur* L.) trees, sulfate uptake was enhanced under $CO_2$ enrichment (650 μl/L) (Seegmüller *et al*., 1996), which indicated that there are genotypic differences in the sulfate absorption response to elevated atmospheric $CO_2$ concentration. In the present study, because of the changes in N and S contents, the N/S ratio decreased in the 5 and 10 mmol N/L treatments under the elevated $CO_2$ concentration, but was not affected significantly by the 20 mmol N/L solution because of the similar change in N and S contents under enriched $CO_2$ concentration. Hesse *et al*.(2004) reported that decrease in the N/S ratio due to diminished N content caused increased synthesis of sulfurous cysteine as a precursor of methionine. Moreover, cysteine and methionine act as effective sulfur donors in thiohydroxamate formation in the syntheses of aliphatic and indolyl GSs (Mikkelsen *et al*., 2002). However, in our study, at the 5 and 10 mmol N/L concentrations, the aliphatic GS concentration increased with the decrease in N/S ratio, but indolyl GS concentration was not affected by the reduced N/S ratio under the elevated $CO_2$ concentration, which agreed with previous results of the effect of elevated $CO_2$ on the GS content of broccoli inflorescences (Schonhof *et al*., 2007). Although indolyl GS concentrations were unchanged under the elevated $CO_2$ concentration, indolyl GS content increased with the increasing N supply, which is in agreement with the findings of Shattuck and Wang (1993) and Kim *et al*.(2002).

Besides the effect of N on GS concentration and composition, plant species is thought to be taken into

account as they experience increasing atmospheric $CO_2$ levels. For broccoli, which is a cultivated derivative of *B. oleracea*, aliphatic GS concentration increased while indole GS concentration decreased under elevated $CO_2$ conditions (Schonhof *et al*., 2007). In a recent study, elevated $CO_2$ (720 μl/L) increased the concentrations of total aliphatic GSs and aromatic GSs and decreased the indole GS concentration in leaves of both transgenic and wild-type oilseed rapes (*Brassica rapa* subsp. *oleifera*) (Himanen *et al*., 2008). However, in *Arabidopsis thaliana* $CO_2$ enrichment did not significantly influence the GS concentration (Bidart-Bouzat *et al*., 2005). Karowe *et al*.(1997) suggested that responses in GS content to elevated $CO_2$ concentration appeared to be species-specific, as in the same experiment total GS concentrations in both young and old mustard (*Brassica juncea*) leaves decreased, whereas GS concentrations in radish (*Raphanus sativus* L.) and turnip (*Brassica rapa* subsp. *rapa*) appeared to be unaffected by $CO_2$ enrichment (724 μl/L). Furthermore, Reddy *et al*.(2004) and Bidart-Bouzat *et al*.(2005) observed that the significant changes in individual GS concentrations were not consistent among cultivars of oilseed rape and *Arabidopsis thaliana* under elevated $CO_2$ conditions, supporting the hypothesis that, in general, the response to elevated $CO_2$ differs among cultivars. Moreover, in Chinese kale bolting stems, the concentrations of aliphatic GS glucoerucin and all individual indolyl GSs were not affected by the elevated $CO_2$ concentration, indicating that the concentrations of individual GSs within a specific GS group (i.e., aliphatic, aromatic, or indolyl) did not change consistently, which agreed with the conclusion that the response to elevated $CO_2$ also depends on the individual GS type (Bidart-Bouzat *et al*., 2005).

## CONCLUSION

The elevated $CO_2$ concentration promoted the growth of Chinese kale in the three N treatments and a high N concentration is beneficial for the growth of Chinese kale. GS concentration was affected by $CO_2$ concentration, N concentration, and $CO_2$×N interactions. Under the elevated $CO_2$ concentration, total GS concentration increased as a result of the increase in aliphatic GS concentration in the 5 and 10 mmol N/L treatments, but there was no significantly difference in the 20 mmol N/L treatment, compared with GS concentration under the ambient $CO_2$ concentration. The maximum total GS concentration was recorded in the 5 mmol N/L treatment under the elevated $CO_2$ concentration. Because of the increase in C content and decrease in N and S contents, the C/N ratio was increased significantly at each N level and N/S ratio decreased significantly in the 5 and 10 mmol N/L treatments. However, changes in the C/N ratio were not a reliable predictor of changes in GS concentration in Chinese kale bolting stems. Changes in N and S contents and the N/S ratio could contribute to the change in GS concentration in the bolting stem of Chinese kale. These results indicate that at an elevated $CO_2$ concentration high N availability promoted the growth of Chinese kale, but reduced the total GS content in bolting stems.

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